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GRANT NUMBER: DAMD17-95-1-5018

TITLE: The Development of Non-Heme-Oxygen Carriers for Use in Blood Substitutes

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REPORT DATE: February 1996

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 1996	Final (15 Dec 94 - 14 Dec 95)

4. TITLE AND SUBTITLE
The Development of Non-Heme-Oxygen Carriers for Use in Blood Substitutes

5. FUNDING NUMBERS
DAMD17-95-1-5018

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

In recent years, the structure and oxygen-binding mechanism of the non-heme protein, hemerythrin, have been greatly clarified. These advancements, coupled with rapid developments in cloning technology and increasing demand for safe effective blood substitutes, suggests that investigation of hemerythrin for this purpose should be closely examined.

We have recently succeeded in cloning and overexpressing functional hemerythrin. Two general questions are being addressed. First, can hemerythrins be found or engineered by chemical modification or site-directed mutagenesis which bind oxygen with P_{50} greater than 20mm Hg and which are at least as stable as hemoglobin at 37 degrees Celsius. Second, are these proteins retained for at least several hours in the vascular system of a suitable animal host, do they have any vasoconstrictive properties and are they toxic when infused.

14. SUBJECT TERMS
PCR - polymerase chain reaction
Hr- hemerythrin

15. NUMBER OF PAGES
34

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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( ) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Donald M Kenney

Principal Investigator's Signature

2/16/96

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I. Introduction and Background

Hemoglobins have been examined for possible usage as O_2 carriers in blood substitutes for at least 50 years (1). Other types of O_2-carrying proteins have apparently been totally neglected in this regard. A search of Index Medicus for the years 1963 through 1991 under the categories of Blood Substitutes produces no references to the use of non-heme O_2-carrying proteins. However, in recent years the structures of these non-heme proteins, hemerythrin (Hr) and hemocyanin (Hcy), have been greatly clarified, as have their mechanisms of O_2 binding (2-4). These insights, coupled with the rapid developments in cloning technology and the increasing demand for safe, effective blood substitutes, strongly suggest that investigations of Hr and Hcy for this purpose should be neglected no longer.

Hr is found in a few phyla of marine invertebrates, occurring most frequently in sipunculid worms. Despite its name, Hr contains no heme group, but rather, a non-heme diiron site. We and others have undertaken detailed characterizations of Hr during the past several years. Although the phylogenic distribution of Hr is relatively limited, there are several dozen species (at least) which are known to contain Hr as the O_2 carrier. The largest quantity of Hr in an animal is invariably found in hemerythrocytes which float freely in the coelomic fluid. Hr from most species consists of an octamer of molecular weight, 108,000. Each of the eight identical subunits reversibly binds one molecule of O_2. We routinely isolate gram quantites of crytstalline Hr from hemerythrocytes of the sipunculid *Phascolopsis gouldii* by a straightforward purification procedure (6). An analogous non-circulating, monomeric protein called myoHr is found in muscle tissues of sipunculids. MyoHr is not contained in erythrocytes and is presumed to be functionally equivalent to myoglobin in mammalian muscle tissues. The molecular weight of myoHr (~13,900) is very close to that of a subunit in the octameric protein (~13,500) as the secondary, tertiary and diiron site structures. High-resolution X-ray crystal structures are available for both Hr and myoHr (2,3) . These high resolution structures will greatly facilitate the planning of chemical and mutagenic changes as well as interpretations of experiments designed to optimize those features required for a viable O_2 carrier in blood substitutes.

Some potential advantages of purified Hr over hemoglobin as a blood substitute include a higher O_2-carrying capacity due to the octameric subunit structure, a higher molecular weight and subunit association constant for the oligomer (8), which should favor an increased residence time in the circulatory system, and no detectable affinity for carbon monoxide, which could make Hr useful in the treatment of carbon monoxide poisoning. Furthermore, nitric oxide binds to *P. gouldii* Hr with an affinity constant ($5 \times 10^6 M^{-1}$(9)) ~4 orders of magnitude less than that for hemoglobin ($3 \times 10^{10} M^{-1}$(10)). Thus, nitric oxide depletion from the reticuloendothelial system could be less of a problem with Hr than with hemoglobin. Finally, the O_2 affinity constant for Hr ($2 \times 10^5 M^{-1}$) is approximately the same as that for stripped hemoglobin ($1.3 \times 10^5 M^{-1}$)(11).

Thus, Hr has a much more inherently selective affinity for O_2 over NO and CO than does hemoglobin. In order to optimize the effectiveness of Hr in a blood substitute, some of its properties may have to be modified. Most Hrs, including that from *P. gouldii* hemerythrocytes, bind O_2 with a P_{50} in the range of 3-5mm Hg at 25°C and Hill coefficient , n~1, both of which are probably lower than optimal for use in a blood substitute. However, ~9 kcal/mole monomer of heat is released upon O_2 binding to deoxy Hr (12); therefore, using the usual equation (13) P_{50} can be predicted to increase to ~7 mm Hg at 37° C. Furthermore, the tentacular Hr from *Themiste zostericola* and the coelomic Hr from *Lingula reevii* are reported to bind O_2 more weakly (P_{50} in the range of 10-26mm Hg at pH 7.5 and 20-22°C) and with greater cooperativity ($n_{max} \geq 2$) (7,8c). These latter two Hrs are available in smaller amounts than is Hr from coelomic hemerythrocytes of *P. gouldii*, but their amounts could be increased by cloning in a suitable host. Alternatively, we may be able to engineer the desired properties into *P. gouldii* Hr by site-directed mutagenesis. We have initiated an effort to sequence as many Hrs and myoHrs as possible in order to lay the foundation for cloning and site-directed mutagenesis. So far we have completely sequenced one new myoHr, that from *P. gouldii* muscle tissue (14) and one new Hr, that from hemerythrocytes of the lingulid brachiopod *Lingula reevii* (15, 16). During the course of this sequencing, we discovered that *L. reevii* Hr consists of two different types of subunits present in equal amounts. This discovery immediately suggests that an $\alpha_4\beta_4$ subunit composition gives rise to the cooperativity in *L. reevii* Hr, since non-cooperative Hrs consist of only a single subunit type (15). Our partial sequencing of Hr from hemerythrocytes of *Glottidea pyramidata*, a brachiopod related to the lingulids found on the U.S.A. gulf coast, also shows two types of subunits with N-terminal sequences strongly resembling the α and ß subunits sequences of *L. reevii* Hr (17,). Thus, several Hrs are available for examination.

Cloning and overexpression of Hrs will definitely be necessary for producing the large amounts of protein necessary for usage in a practical blood substitute as well as for introduction of desirable site-directed mutations. We have overcome a potential problem in cloning and overexpression of Hr by demonstrating high-yield reconstitutions of functional Hr and myoHr starting from the apoproteins in guanidinium chloride (5). No published report of a recombinant Hr has appeared. A report of a recombinant myoHr from a synthetic gene which duplicates the amino acid sequence of *T. zostericola* myoHr has appeared, and this recombinant myoHr, after purification from the bacterial cell extract, was found to contain an intact diiron site (18). We have recently cloned and overexpressed the myoHr gene from *P. gouldii* muscle tissue (19). This purified recombinant myoHr too was found to contain an intact diiron site. Probably the most thoroughly characterized of any Hr is that from the sipunculid worm *Phascolopsis gouldii*, and it is from this organism that we have recently obtained a recombinant Hr. Cloning of the *P. gouldii* Hr gene was accomplished by the PI in the laboratory of Professor Robert L. Robson at

the University of Georgia. We chose to reverse transcribe total RNA isolated from *P. gouldii* hemerythrocytes, then use the PCR on the resulting cDNA to specifically amplify the Hr gene. The RNA isolation kit from Stratagene was used to isolate RNA from *P. gouldii* hemerythrocytes and the GeneAmp RNA PCR kit from Perkin-Elmer Cetus was used for reverse transcription and PCR. Oligonucleotides DMK2 and DMK3 were used for the PCR step.

PstI rbs ↓

DMK2 5' GCTGCAGTAAGGAGGTTTAACATG GGN TTY CCN ATH CCN GAY 3'

HindIII

DMK3 5' ATGCAAGCTTA DAT YTT NCC YTT RTA YTT 3'

DMK2 and DMK3 were designed to overlap the 5' and complementary 3'ends, respectively, of the most ambiguous reverse translation of the published *P. gouldii* Hr amino acid sequence (Y=C or T; W=A or T; R=A or G; D=A, G or T; H=A, C or T; N= G, A, T or C). The indicated PstI and HindIII restriction sites were also incorporated and the extra bases upstream of these restriction sites were included to ensure efficient cleavage of the resulting PCR product (20). The indicated ribosome binding site (rbs) and start (↓) and stop (*) codons were also incorporated. The PCR product was ligated into pBluescript KS+, and the resulting plasmid, pDK1, was subjected to chain termination DNA sequencing (21) using ^{35}S-ATP and Sequenase (United States Biochemical Corporation) according to the manufacturer's instructions. A DNA sequence whose translation matches that of one of the genetic variants published for *P. gouldii* hemerythrocyte Hr was obtained. We achieved massive overexpression of the *P. gouldii* Hr gene using the T7 RNA polymerase/promoter system of Tabor (2) . This system requires two plasmids: pGP1-2, which contains the T7 RNA Polymerase gene under control of a promoter that is repressed by a temperature-sensitive repressor, and pT7-7, which contains the T7 RNA polymerase promoter and ribosome binding site followed by a polylinker for insertion of the cloned gene. A typical temperature sequence for overexpression in this two-plasmid system is 42°C for 30 minutes, during which synthesis of the T7 RNA polymerase is induced, followed by incubation at 37-40°C for 90 minutes, during which the gene inserted into pT7-7 is overexpressed. The highest levels of overexpression are achieved when the start codon of the gene is inserted into NdeI site of pT7-7, because of its optimal placement downstream of the ribosome binding site and T7 promoter. Therefore, the PCR with the appropriate oligonucleotide primers was used to introduce an NdeI site (CATATG) at the start codon of the Hr gene. The resulting Hr gene, now containing NdeI/HindIII termini, was ligated into pT7-7, thereby generating (presumably identical) plasmids, pDK4-1 and pDK4-2. These plasmids were transformed into K38(pGP1-2). The resulting clones were induced at 42°C for 30 minutes followed by 90minute incubation at 40°C. We estimate that induction under these conditions

produces approximately 100mg of cloned Hr per liter of culture! Analysis of sonicated bacterial extracts indicates that the cloned Hr is contained in inclusion bodies when induced. Dissolution of the washed inclusion bodies in 6M GdmCl and reconstitution according to our published procedure (5) and addition of azide led to the very stable metHrN3 adduct. No other purification steps were needed!

Note that oligonucleotides DMK2 and DMK3 (listed above) encode the N- and C-terminal six residues, respectively, of *P. gouldii* Hr. It has been established that these residues are highly conserved in all Hrs, and, therefore, single consensus sequences for the N- and C-terminal six residues would probably not significantly affect the function or stability of any Hr. If so, then the PCR could be used with oligonucleotide primers either identical or very similar to DMK2 and DMK3 to amplify the cDNA encoding Hrs from any organism, even when the amino acid sequence is not known. Finally, it is noteworthy that no other laboratory in the world has our experience with Hr combined with the ability to clone and overexpress these proteins. Therefore, we are uniquely qualified to examine the usage of these proteins in blood substitutes.

An analogous approach can be envisioned for cloning and overexpression of the copper O_2-carrying protein, hemocyanin(Hcy), which is found in arthropods and mollusks. Some properties of Hcy may already be close to those required for a blood substitute (23). For example, Hcys occur exclusively as extracellular proteins which circulates throughout the organism. At least one Hcy retains functionality after storage as a freeze-dried powder with sucrose (24). Hcy binds CO approximately an order of magnitude less strongly than O_2 (23a); nitric oxide, in contrast, slowly oxidizes deoxyHcy (23a). Although some Hcys are reported to by highly immunogenic, this immune response is invariably induced by combination with adjuvant, and, therefore, purified Hcy by itself, may not provoke the same immune response. Furthermore, all known Hcys are glycosylated, whereas, recombinant proteins overexpressed in *E. coli* are not glycosylated. Therefore, if the immune response is stimulated by the surface carbohydrate moieties, then recombinant Hcys may be much less immunogenic than the native Hcys.

No reports of cloned and overexpressed Hcys have yet appeared. Hcys from *Limulus polyphemus* (horseshoe crab) and *Octopus dofleini* (giant Pacific octopus) appear to be good candidates for cloning and overexpression. These two Hcys are representative of arthropod (*L. polyphemus*) and molluscan (*O. dofleini)* Hcys and have subunit molecular weights of ~75,000 and ~350,000, respectively (25). *L. polyphemus* Hcy associates into oligomers containing up to 48 subunits in vivo, although aggregation usually requires Ca^{2+} and is highly dependent on ionic strength and pH (26). Therefore, the aggregation state of the recombinant protein is difficult to predict. The isolated *L. polypheus* Hcy subunits in the absence of Ca^{2+} are quite stable; each subunit contains one dicopper site, which binds O_2 with P_{50} ~ 2 mm Hg at pH 7 and 20°C(26).

This P_{50} is probably too low for an effective blood substitute. However, -9 to -14 kcal/mole heat is released upon O_2 binding to *L polyphemus* Hcy (27); therefore, P_{50} at 37°C can be estimated to be ~6 mm Hg. Additional attractive features of *L. polyphemus* Hcy is that its high resolution X-ray crystal structure, including the O_2 binding site, is available (28,29), and a complete amino acid sequence of subunit II (the same subunit whose X-ray crystal structure was determined) is also available (30). Furthermore, mRNA coding for the *L. polyphemus* Hcy subunits has been isolated from total RNA and translated in a cell-free system (31). Proteins immunologically identical to native Hcy were isolated from this cell-free translation. These results all suggest that cloning and overexpression of *L. polyphemus* Hcy would be feasible using methods completely analogous to those described above for Hr.

The *Octopus dofleini* Hcy subunit consists of seven O_2 binding domains, each of which contains two copper ions and has a molecular weight of ~50,000. These domains are sequentially N- and C-terminally linked like "beads on string" , which provides an attractive possibility for controlling molecular weight and number of O_2 binding domains. cDNA encoding the M_r 45,000 carboxyl-terminal domain of Hcy from *Octopus dofleini* has been cloned in *E. coli* and sequenced (32). No report of overexpression of this cDNA has appeared, but the corresponding protein domain can be prepared by trypsinolysis of native *O. dofleini* Hcy, and this trypsin fragment was shown to bind O_2 with P_{50} = 50mm Hg at pH 7.65 and 20°C (25). cDNA coding for two adjacent O_2 binding domains of *O. dofleini* Hcy has also been identified and sequenced (33). Therefore, for *O. dofleini* Hcy, we can use techniques analogous to those described above for Hr to amplify and overexpress the genes coding for the at least three O_2 binding domains.

There seems to be no clear way to completely suppress reactions of Hr and Hcy with NO without simultaneously affecting O_2 binding. For example, one expects that mutations which lower O_2 affinity will also inhibit binding of or reaction with NO. Therefore, rather than measure NO binding in vitro, we propose to directly measure the putative in vivo consequences of NO binding, namely vascular reactivity, and use hemoglobin solutions as standards of comparision.

II Body

The general experimental chronology for each Hr and Hcy would be: i) isolation of total RNA from the appropriate tissue, ii) amplification of the cDNA, cloning and sequencing of the gene, iii) overexpression using a T7 RNA polymerase/promoter system on a 1-liter culture scale, iv) isolation and purification of the overexpressed protein, v) measurements of molecular weight, metal content and P_{50} fo the overexpressed proteins, vi) tests of protein stability at 37°C, vii)

site-directed mutagenesis, if necessary, viii) scaleup of protein production, ix) tests for vasoconstriction, and x) assays of retention times and renal toxicity.

Hrs from the following sources (at least) will be investigated: *P. gouldii* hemerythrocyte, *L. reevii* hemerythrocytes, *G. pyramidata* hemerythrocyte, *T. zostericola* tentacles. The reasons for choosing these particular Hrs have been discussed above; many more are potentially available. *P. gouldii* are available live from the Marine Biological Laboratory, Woods Hole, MA. *L reevii* are available from the Deep Blue C. Co., Honolulu, HI. A letter stating the willingness of Colin Lau to collect and ship these animals is included in the addenda. *G. pyramidata* and *T. zostericola* are available Gulf Specimens, Inc., Panacea, FL and Pacific Biomarine Labs, Inc., Venice CA, respectively. *L. polyphemus* will be obtained from horseshoe crabs purchased live from Woods Hole, MA *O. dofleini* will be obtained from Pacific Biomarine Labs, Venice CA. In principle only one shipment of each of these animals should be necessary; after obtaining the genes for the Hrs and Hcys, we should have no further need for the animals themselves.

A. Isolation of Total RNA and amplification of cDNA

In the case of Hrs, total RNA will be isolated from hemerythrocytes of the various animals. In the case of Hcys, total RNA will be isolated from behind the compound eye (a site of Hcy biosynthesis) of the horseshoe crab, *Limulus polyphemus* or from the branchial gland *O. dofleini*. The isolation procedure will follow that used for *P. gouldii* hemerythrocyte total RNA as described above or the published procedures for Hcy (31,32) but using the total RNA isolation kit from Stratagene. Amplification of the genes for the Hrs and Hcys will be accomplished using the RNA PCR method discussed for *P. gouldii* Hr in Section I. Nucleotide sequencing of the PCR products will use the standard dideoxy chain termination procedure (21). Degenerate oligonucleotides analogous to DMK2 and DMK3 wil be used for the PCR. Note that we expect to amplify at least two different genes for the *L. reevii* and *G. pyramidata* Hrs in the PCR, because these Hrs contain two different types of subunit (cf. Figure 3). Figure 3 shows tha the N-terminal seven residues in the amino acid sequences of the *L. reevii* Hr α and ß subunits are identical to each other except for the first and seventh residue, and the C-terminal seven residues of both *L. reevii* Hr subunits are identical to each other. Therefore, we plan to carry out two separate RNA PCRs, one using a 5' oligonucleotide primer for the α subunit gene and a second using the corresponding primer for the ß subunit gene. These primers would also contain degeneracies coding for the most ambiguous reverse translations of the amino acid sequences. These oligonucleotides are listed below.

5' α subunit oligo 5' TATACATATG GTN AAR GTN CCN GCN CCN TTY 3'

5' ß subunit oligo 5' TATACATATG ATG AAR GTN CCN GCN CCN TAY 3'

3' α/ß subunit oligo: 5' ATGCAAGCTTA NAR YTT NCC YTT RTA YTT RAA 3'

The most probably reverse translations (using the Genetics Computer Group software) of the α and ß subunit amino acid sequences show no internal NdeI or HindIII restriction sites. As described in Section I, an NdeI site is needed for maximal overexpression in the T7 RNA polymerase/promoter system(22). Several clones of the PCR products may have to be sequenced in order to identify those containing the correctly coded genes for both subunits. For overexpression, we plan to sequentially link the genes for the α and ß subunits and insert the linked pair of genes into pT7-7 (cf. Figure 5). The linker between the α and ß subunit genes would contain a ribosome binding site, and this linkage can be accomplished by a standard PCR procedure (37).

B. Cloning and Overexpression

Cloning and overexpression of the genes obtained from the PCR will be accomplished in the T7 RNA polymerase/promoter system, which we used successfully for *P. gouldii* Hr and which is illustrated in Figure 5 (22). Our familiarity and success with this system means that we can initiate studies of overexpression very quickly after amplification of the genes for the various Hrs and Hcys. A second potential advantage is that the T7 RNA polymerase/promoter system uses heat rather than an added chemical to induce overexpression of the cloned gene; scaleup will, therefore, be cheaper. If necessary, we will use an alternative to heat induction developed by Studier et al. (38). This system uses a strain of E. Coli, BL21DE3 (available from Novagen, Inc.), which contains an integrated (chromosomal) T7 RNA polymerase gene under control of the *lac* promoter. In this case, we would transform BL21DE3 with pT7-7 containing the inserted Hr gene and induce overexpression by addition of the saccharide, IPTG. Such a system has been used successfully by others to overexpress proteins containing heme and flavin prosthetic groups (39).

The *L. polyphemus* Hcy gene will be obtained and overexpressed analogously to the procedure described above. From the known amino acid sequence of *L. polyphemus* Hcy subunit II (30), degenerate oligonucleotides coding for the N- and C-terminal portions of the *L. polyphemus* Hcy gene can be designed, synthesized and used in the RNA PCR to generate first, the cDNA and then the amplified Hey gene. The most probable reverse translation (using the Genetics Computer Group software) of the *L. polyphemus* Hcy subunit amino acid sequence shows no internal Ndel restriction site. Therefore, the oligonucleotide primer for the 5' end of the gene in the RNA PCR can contain an Ndel site at the start codon. In the case of *O. dofleini* Hcy, the cDNA sequences for both a single O_2 binding domain and two adjacent O_2 binding domains have been published (32,33). Therefore, non-degenerate oligonucleotides exactly matching the N-terminal and complementary C-terminal portions of the cDNAs can be used in the PCR. Thus, a gene containing either one or two O_2 binding domains can be amplified and overexpressed starting from total RNA. The single-domain cDNA contains an internal Nde l site

but no internal Sma 1 site; therefore a SmaI site can be inserted at the 5' end of the single-domain cDNA by the PCR. When inserted into pT7-7, the gene can then be expressed with the five-residue N-terminal extension, ARIRAR, in order to place the start codon optimally downstream of the ribosome binding site. This five-residue extension is coded for on the polylinker of pT7-7. The two-domain cDNA does not contain an internal NdeI site; therefore, an NdeI site can be inserted into the start codon of this two-domain cDNA be PCR. Once these amplifications are accomplished, cDNAs encoding these domains can be linked together, if necessary, by a standard PCR procedure (37) to construct a gene encoding the desired number of O_2 binding domains.

An enzyme closely related to molluscan Hcys, namely, tyrosinase from *Streptomyces antibioticus*, has been cloned and overexpressed in E. coli using the same T7 RNA polymerase/promoter system illustrated in Figure 5 (40). This protein contains a copper site very similar to that in molluscan Hcys (23a). In order for the tyrosinase to be overexpressed in active form, another gene, namely ORF438 from *Streptomyces antibioticus* had to be included upstream of the tyrosinase gene. ORF 438 encodes a protein which is apparently required for insertion of copper into *S. antibioticus* tyrosinase. Therefore, simultaneous overexpression of *S. antibioticus* ORF438 and the Hcy genes may facilitate insertion of copper into the recombinant Hcy. A plasmid containing *S. antibioticus* ORF438 has been kindly supplied to us by Dr. Guiy della-Cioppa of Biosource Genetics Corporation and will be inserted upstream of the Hcy genes, if necessary. A letter from Dr. della-Cioppa and a plasmid map containing ORF438 are contained in the addenda.

C. Isolation and Purification of Recombinant Proteins

Optimal growth conditions and purification procedures for the overexpressed proteins will have to be worked out by trial and error using standard techniques, but detection of the overexpressed proteins should not be difficult, since the T7 RNA polymerase/promoter system typically results in overexpression to at least 10% of total cell protein (22). If necessary, Hrs will be reconstituted with iron by our standard procedure (5). Measurements of molecular weight can be done either by SDS-PAGE or for loigomers by gel filtration chromatography. Both Hr and Hcy have distinctive and characteristic UV/vis spectra in their oxygenated forms, which will be used to identify and quantitate the diiron and dicopper sites in these proteins (5,23a). Metal contents of the proteins will be quantitated by ICP-AE spectrometry. O_2 affinities will be measured tonometrically using absorbance at 500nm for Hr (5) and 340 nm for Hcy (23,24). We have used the tonometer and described the procedure previously for P. gouldii Hr (41). Standard Hill plots will be used to quantitate cooperativity of O_2 binding. Because of the lengthy equilibration times, the O_2 binding curves will probably be determined at 20-25°C. Tests of stabilities at 37°C can be accomplished spectrophotometrically using decreases in

absorbance at either 500nm (Hr) or 340nm (Hcy) to monitor O_2 binding site losses. Bovine oxyhemoglobin (Sigma Chemical Co.) will be used as the standard of comparison for stability at 37°C.

D. Site-Directed Mutagenesis

Since P_{50} for the *P. gouldii* hemerythrocyte Hr may be too low even at 37°C, site-directed mutations aimed at lowering O_2 affinity may be required. Such mutations are the subject of a recently funded NIH grant (GM40388) and would not be duplicated here. Rather, mutations aimed at stabilizing *P. gouldii* Hr at 37°C would be pursued. Since the enthalpy of association of *P. gouldii* Hr. subunits is slightly negative (-2.6 kcal/mole of monomer), raising the temperature to 37°C is likely to shift the equilibrium slightly towards dissociated subunits, and the dissociated subunits are probably less stable to denaturation than is the octamer. Therefore, site-directed mutations whichlead to increased intersubunit interactions in *P. gouldii* Hr will be pursued. Examination of the octameric structure of *P. gouldii* Hr reveals no obvious way of covalently linking subunits by recombinant DNA techniques while conserving the native quaternary structure. Therefore, we will attempt to increase the number and/or strength of non-covalent intersubunit interactions by site-directed mutagenesis. Because of the likely difficulty of screening large numbers of mutated proteins for the desired properties, random mutagenesis is probably not the optimal approach, at least initially. Residues targeted for mutation to increase intersubunit interactions will be limited to non-conserved residues (cf. Figure 3), because non-conserved residues must not be absolutely required for O_2 binding. However, residue pairs engaging in non-covalent intersubunit interactions (i.e., hydrogen bonds and salt bridges) in native *P. gouldii* Hr have already been delineated from the X-ray crystal structure and are identical to those residue pairs in T> dyscritum Hr (3c, 34). Therefore, these residue pairs will be targeted initially. One possibility is a Cys50→Ser mutation. Cys50 is known to be involved in a hydrogen-bonded intersubunit interaction with the carboxylate of Asp23 (3c, 34). These interactions are illustrated in Figure 1. Two such interactions occur between A and B helices across the Q two-fold rotation axis, and there are a total of eight such interactions in the octamer. The interaction with Asp23 could still occur and perhaps even be stronger with the serine hydroxyl side chain, especially if closer intersubunit packing occurs due to the substitution of sulfur with the smaller oxygen. The Cys50 S is known to be in van der Waal's contact with surrounding residues (34), and chemical modification of Cys50 with mercurials is known to lead to subunit dissociation (35,36). Closer intersubunit contacts resulting from the Cys50→Ser mutation could also strengthen other known pairwise intersubunit interactions with the Asp23 carboxylate, including salt-bridges with the side chains of Arg49 and Lys 53 (3c,34). Additional benefits may arise from the fact that Cys50 is the only Cys residue in *P. gouldii* Hr. The Cys50→Ser mutation removes the possibility of disulfide cross-linking between dissociated

subunits at 37°C. Removal of the possibility of disulfide cross-linking might also increase the yield of soluble recombinant *P. gouldii* Hr in *E. coli*. Space limitations prevent the discussion of several other planned site-directed mutations.

Mutations aimed at increasing the stability of the individual subunits of *P. gouldii* Hr will also be pursued. In this case the aim is to increase or strengthen interactions between the N- and C- terminal regions within each subunit via salt-bridge or hydrogen bonding interactions; once again the targeted residues are not strictly conserved and, therefore, must not be absolutely required for O_2 binding. Space limitations prevent discussion of specific mutations, but there are several possibilities. In all cases, site-directed mutagenesis would be carried out using a standard PCR method (40). This method has been chosen over several others because of its convenience. Unlike most other mutagenic protocols, the PCR method would not require transfer of the Hr genes between another vector and pT7-7. All mutated genes will be sequenced to ensure their correctness.

E. Scaleup of Protein Production

Scaleup of all protein overexpression will use one of the T7 RNA polymerase/promoter system described above and will use up to 400-liter fermenters for growth of bacterial cultures and induction of protein overexpression. If inclusion bodies are obtained, then a large-scale reconstitution procedure will be developed (5). The Bio-Pilot FPLC from PharmaciaLKB will be used for large-scale purifications of soluble recombinant Hrs and Hcys. The ion-exchange columns on this FPLC can process liter volumes of extract in one pass.

F. Biologic Evaluation of Hemerythrin as an Oxygen Transporter

The experiments described below will be carried out under the supervision of Dr. Clarence Rawlings. The experiments are described for Hr, but similar experiments on Hcy will also be carried out. Dr. Rawlings has completed and published using the surgical and laboratory procedures presented in this protocol.

1. Duration of hemerythrin in the circulation

Objective: Determine the in vivo half-life of hemerythrin in the circulation and its renal excretion.

Background: It is important to determine how long hemerythrin remains in the circulation. In acute blood loss in a previously healthy patient, several days are required prior to bone marrow production of new red blood cells. It would be ideal that a foreign protein being used for oxygen transport be administered only once. Preferably, hemerythrin would remain in the vascular system until the patient's natural hemoglobin was again able to transport oxygen. Young pigs were selected for both the pharmacokinetic and toxicity studies as it was anticipated

that hypovolemic shock studies will be performed in later studies. Pigs have been used as a hypovolemic model in previous resuscitation studies, particularly at Letterman Army Institute of Research. Fortunately, we have considerable experience in using pigs for anesthesia and surgical research. Appropriate facilities and laboratory personnel are available. Animal Use Proposals have been submitted to Animal Care and Use for both Studies.

Methods: Eight young (15kg) female pigs that have no abnormalities on physical examination, CBC, serum chemistry profile, and urinalysis will be studied. The pigs will be anesthetized with acepromazine (0.1 mg/kg, IM), telazol (emg/kg, IM), and halothane via an endotracheal tube. Catheters will be placed into the right atrium and the urinary bladder for obtaining samples determination of hemerythrin in the circulation. The right atrial catheter will be positioned using a facilitative maneuver to place a 14g needle in the right side of the thoracic inlet. A catheter will be passed through the needle and then through a subcutaneous tract to exit in the dorsum of the neck. A Foley catheter will be placed into the bladder via the urethra. The pigs will be recovered from anesthesia. On the day following instrumentation, the pigs will be placed in a stanchion in order to closely monitor their behavior and provide access to catheters. Baseline venous blood and urine samples will be obtained. And intravenous catheter will be inserted into an ear vein for infusion of hemerythrin. Approximately 30% of the blood volume (400ml) will be removed prior to replacement with a volume of normal saline containing 50mg/ml of hemerythrin. Blood will be withdrawn from the right atrial catheter and hemerythrin will be infused into the ear vein catheter. Following completion of the infusion, 5ml blood samples will be obtained from the atrial catheter at 0.5,1,2,3,4,5,6,24,48, and 72 hours. Urine will be sampled at 1,2,3,4,5,6,24,48, and 72 hours. Samples will be frozen. The hemerythrin in the blood and urine samples will be detected and quantitated by SDS-PAGE/Western blots of the blood and urine samples will be detected and quantitated by SDS-PAGE/Western blots of the blood and urine samples collected from infused animals. Rabbit anti-Hr and anti-Hcy antibodies will be obtained from Berkeley Antibody Co., whom we will supply with the Hrs and Hcys. Western blots will be obtained by the standard second antibody/alkaline phosphatase method (42), using either the NBT/BIP substrate, which generates a purple color on nitrocellulose, or the chemiluminescent substrate, AMPPD, followed by exposure of the nitrocellulose to film. We have been able to easily detect 50ng of Hr on Western blots (using NBT/BCIP substrate) in *E. coli* extracts containing a few protein percent of added native Hr. The chemiluminescent substrate is typically an order of magnitude more sensitive. The Western blots with either substrate will be quantitated densitometrically and plotted versus time following injection. The biological half-life will be determined.

Interpretation: Characterization of how long hemerythrin remains in the circulation is important if this oxygen transporter can fill the void between acute blood loss and endogenous

production of red blood cells. Later studies would be done to determine if the half-life is modified by its use for resuscitation of hypovolemic shock.

2. Potential toxicity of hemerythrin

Objective: Determine effects of hemerythrin on hematologic, renal, hepatic, pulmonary, and cardiovascular systems.

Background: Free hemoglobin in high concentrations within the vascular system can produce hepatic and renal complications during their attempt to process and excrete the hemoglobin. Cardiopulmonary responses might also be expected. A potential complication for an oxygen transporting protein is the binding with NO (Endothelial Derived Relaxing Factor) or even free radicals. Binding of NO might have a vasoconstrictive (hypertensive) effect, whereas binding of the superoxides might be vasodilatory. The cardiovascular effects will be determined from pressure and flow measurements. Foreign proteins may initiate an anaphylactic response. The use of pigs as a model was presented above. An anaphylactic reaction can be readily observed in white skinned pigs.

Methods: Young female pigs will be used as in the hemerythrin half-life studies above. Anesthesia will be done as before in order to place catheters into the pulmonary artery, aorta, and urinary bladder. The pulmonary arterial catheter will be a thermodilution catheter for measurement of cardiac output and pulmonary arterial pressure. The aortic catheter will be placed either via the carotid artery by surgical cutdown or preferably the technique previously described for a translumbar approach to the abdominal aorta caudal to the renal arteries. This later approach, which was developed at Letterman, reduces the likelihood of catheter induced emboli being distributed to the kidneys. On the day following instrumentation, the pigs will be stanchioned and baseline measurements will be taken. These will include the clinical pathologic samples, cardiac output, heart rate, respiratory rate, pulmonary arterial pressure, aortic pressure, systemic arterial blood gas determination, and behavior. Thirty percent of the blood volume will be removed and replaced with either lactated Ringer's solution, matched blood, or normal saline with hemerythrin (50mg/ml). Each of the three groups of pigs will consist of eight pigs. Hemodynamic measurements done before hemerythrin infusion will be done at 0.25,0.5,1,2,3,4,5,6,24,48, and 72 hours after completion of infusion. Arterial blood gas samples will be done at 1,3,6, and 24 hours. Samples for CBC, serum chemistry profile, and urinalysis at 6,24,48, and 72 hours. Complete physical examination will also be done at these time intervals. Bromosulphathalein and exogenous creatinine clearances will be done at 72 hours. The pigs will be euthanitized and complete necropsies performed. Organs to be sampled and examined histologically include heart, lung, liver, kidney, and lymph nodes. These will be examined by one of the board certified veterinary pathologists at the College of Veterinary Medicine. The

specific colleague will be included in protocol development and tissue interpretation once funding is determined.

Interpretation: Massive blood loss and resuscitation with hemerythrin will produce a challenge to the body for oxygen transportation and the elimination of hemerythrin, especially if the half-life is short. If there are minimal cardiopulmonary and anaphylactic effects, then hemerythrin should then be used in hypovolemic shock studies. The latent effects on the liver and kidneys due to massive metabolism and elimination of hemerythrin can restrict and modify use of any novel oxygen transporter.

3. Effects of hemerythrin on vascular reactivity

Objective: Since hemerythrin might bind more than just oxygen, its impact on binding with NO(Endothelial Derived Relaxing Factor) [44] and even free radicals. Endothelial Derived Relaxing Factor provides vasodilation and free radicals tend to increase vascular tone and resistance. Binding of NO might have a vasoconstrictive (hypertensive) effect, whereas binding of the superoxides might be vasodilatory. Due to their short biological half-lives, particularly with NO, these effects may not be important. These effects can be evaluated by perfusion of various concentrations of hemerytrhin through tissue baths containing systemic arterial rings. The use of vasoactive agents should provide information about any potential vascular effect from hemerythrin. The presence of superoxide, which is a metabolite of catecholamines, can be measured in the tissue bath by using chemoluminescence.

Methods: Because of our previous experience and their availability, dogs will be the source for these studies. Samples of the common carotid and femoral arteries will be placed into chilled, aerated (95%O_2, 5% CO_2) Krebs Henseleit solution of the following composition (in mM): NaCl 118.2; KCl 4.6; $MgSO_4$ 1.2;KH_2PO_4 1.2;$CaCl_2$ 2.5; $NaHCO_3$ 24.8; and dextrose 10.00. The arteries will be cut into 4mm rings and suspended in 10ml tissue baths filled with aerated Krebs Henseleit solution maintained at 37°C. Isometric measurements of the vascular rings will be made with a Grass FT 03 force displacemnt transducers and recorded on a Grass Polygraph as changes in grams of force. Four to six vascular rings will be obtained from each dog. Following an equilibration period of approximately 15min, optimal resting tension will be determined for each vascular ring by the maximal response to norepinephrine (5×10^{-7}M). Each vascular segment will then be equilibrated for an additional 90 min at the optimal resting tension (~2 grams) for all dogs. During the equilibration period, the vascular rings will be washed every 15 min with fresh oxygenated buffer solution to prevent the accumulation of metabolic waste products. Concentration-response curves will then be determined for 5, 10, 25, and 50 mg/ml of hemerythrin in normal saline. Concentration-response curves to the following agents with and without 50mg/ml of Hr will also be determined: acetylcholine, norepinephrine, histamine, and serotonin (obtained as HCl salts from Sigma Chemical Co.). A collaborator, Dr.

Randall Tackett, a faculty member in the UGA College of Pharmacy, has agreed to assist us in measuring superoxide concentrations in the tissue baths. For comparison protein concentrations will also be examined. From the cumulative dose response curves, data will be expressed as the percent of maximum response. The maximum contractions of blood vessels will be measured and reported as grams of tension per cross sectional area.[45] Probit analysis will be used to calculate EC^{50}'s, which will be presented as geometric means. Changes in EC^{50}'s and tension will be compared statistically with significance considered at $p \leq 0.05$

Interpretation: The invitro vascular reactivity studies will be more discriminating in its characterization of hemerythrin's effect on the vasomotor equilibrium of arteries. In addition to vasomotor studies to determine if hemerythrin binds NO and free radicals, the potential for these substances to reduce the oxygen binding capacity of hemerythrin by oxygen saturation studies. These will be done on hemerythrin before and after exposure to vascular rings, especially those exposed to cathecholamine infusions.

III. Conclusions

While hemogloblins have been extensively examined for possible use as oxygen carriers in blood substitutes, the non-heme iron O_2-carrying proteins, hemerythrin and myohemerythrin, have been totally neglected in this regard. In recent years, however, the structures of hemerythrin and myohemerythrin and their mechanism of reversible O_2 binding have been greatly clarified. We have also recently succeeded in cloning and overexpressing functional hemerythrin and myohemerythrin. These developments coupled with the increasing demand for safe, effective blood substitutes, strongly suggest that investigations of hemerythrin and myohemerythrin for this purpose should be pursued. Two general questions are addressed in this proposal. First, can hemerythrins be engineered by either site-directed mutagenesis or chemical modification to bind O_2 with $P_{50} \geq 20$ mm Hg and to be at least as stable as hemoglobin at 37 °C? Second, will these engineered proteins be retained for at least several hours in the vascular system of a suitable animal host, will they have any vasoconstrictive properties, and will they be toxic when infused? Procedures for investigating each of these questions are described.

We have cloned and overexpressed the genes for both Hr and myoHr from the sipunculid worm, *Phascolopsis gouldii* in *E. coli*.[46] These overexpressions make available gram quantities of protein, and afford the opportunity to design and express site-directed mutants. The recombinant Hr is overexpressed as insoluble inclusion bodies which can then be resolubilized in guanidinium chloride. Slow, anaerobic dilution of the resolubilized Hr in the presence of ferrous iron affords large amounts of extremely pure, functional protein. MyoHr is overexpressed in *E. coli* as a soluble, functional protein.[46] The University of Georgia operates a fermentation facility with 20-, 50-, and 100-liter fermentors which we can use for large scale growths of the overexpressing *E. coli* strains. These procedures are now routine in our laboratory for the wild-type proteins. We have been and will continue to devote much of our time to testing of the wild-type Hr and myoHr for toxicity, circulatory retention time and vasoconstrictive properties, as orginally described in the parent proposal. Those mutated/chemically modified Hrs and myoHrs that appear to have the desired properties will then be subjected to the physiological studies listed

above. Parallel studies on both Hr and myoHr should provide greater insights into structure/function relationships and faster optimization of the desired properties than would studies on either one alone.

We have already prepared one site-directed mutant of both Hr and myoHr, namely, Tyr109Phe, by standard molecular biological methods.[46] We have established that both the wild-type and the Tyr109Phe mutant of Hr bind O_2 reversibly. The absorption spectra of these two recombinant Hrs in their oxygenated forms (labelled oxyHr and oxyHrY109F, respectively) are shown in Figure 1. The P_{50}s of these Hrs and myoHrs will be measured using a tonometric apparatus previously used in our laboratory to measure O_2 affinties of Hr.[47] Rates of autoxidation to the met forms will be determined spectrophotometrically by monitoring the decrease in the 500 nm component of the oxyHr spectra over time (Figure 1). Results from these experiments will enable us to make more intelligent decisions about which of the proteins' features need to be modified in our pursuit of a blood substitute and help us to target future mutations.

We have found that recombinant oxyHr is soluble to at least 50 mg/ml and stable in Krebs-Henseleit buffer (which is commonly used for vascular reactivity studies). Tests for the presence of endotoxins in Hr preparations are currently being performed in our laboratory, using a commercially available kit. A collaborator, Dr. Randall Tackett from the UGA College of Pharmacy, is conducting studies on the vascular reactivity of the protein using segments from canine carotid and femoral arteries. Pending the results of these experiments, the protein's retention in the circulatory system will be examined. Since Hr is a protein of appreciable size, the rate of clearance by the kidneys should be acceptable. If, however, renal clearance or immunoreactivity proves problematic, functionalization of the protein with polyethylene glycol derivatives will be pursued.[48,49,50] A variety of PEG-derivatives are available which readily react with the free amino group of exposed lysine residues, of which Hr has several. The resulting derivatized protein has a larger mass but often has virtually unchanged functional properties. PEG-modified proteins have exhibited greater retention times in the blood and a

slower immune response than the unmodified proteins.[51] All systems appear to be in place for construction and testing of mutated/chemically modified Hrs and myoHrs as O_2 carriers in blood substitutes.

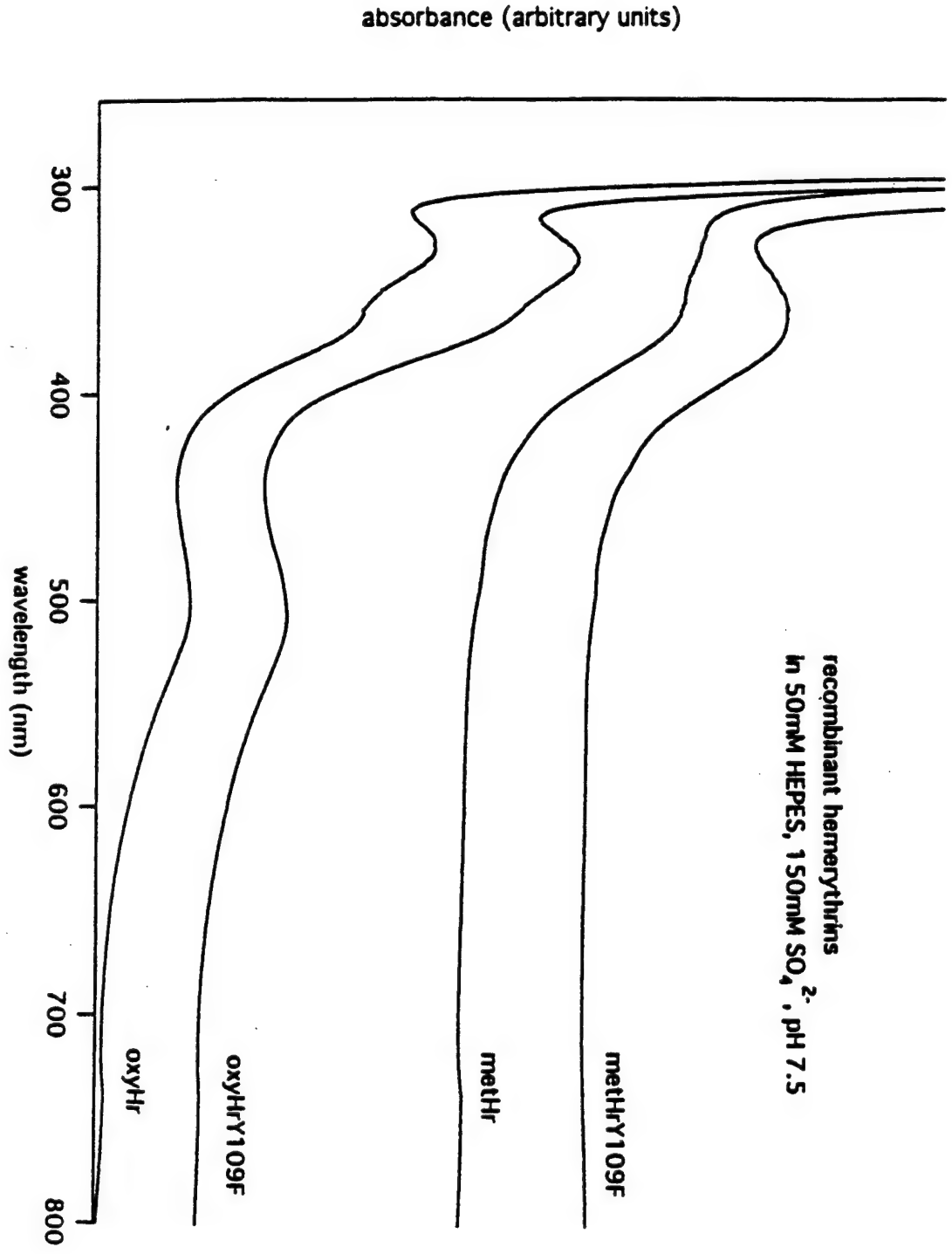

Figure 1. Near-UV/vis absorption spectra of oxy and met forms of recombinant Hrs produced in the Kurtz laboratory.

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V. Appendix.

HEMERYTHRIN PREPARATION:
INDUCTION:

50 ml LB (luria broth, 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter dd H_2O) in 250 ml flask is autoclaved. 50 ul stock ampicillin (100mg/ml) added to flask before innoculation. E. coli (BL21) colonies which contain the pdk4-1 plasmid are picked by sterilized loop and swirled into flask to disperse bacteria throughout media. The innoculated media is incubated at 37° C 250 rpm shaker overnight. Next AM, one liter LB/ampicillin cultures are innoculated with aforementioned 50 ml cultures. Incubation continues at same conditions.

Absorbance at 600 nm is checked hourly. At an OD of 0.8 to 1.0, 4 g lactose is added to induce the production of Hr protein. After induction the absorbance should increase steadily. Cultures are harvested 2 to 3 hours or when absorbance is between 4 and 5.

Whole cell pellets are obtains by centrifugation at 8,000 rpm for 20 minutes utilizing a JA10 rotor at 4 C (Beckman J2-21). Cell pelletes are stored at -80 C. For cell lysis, whole cells are thoroughly resuspended into 50 mM Tris + 2 mM EDTA buffer (pH 7.5) 10 ml buffer per gram whole cells is sufficient. Suspension is next lysed utilizing a 550 sonic dismembrator (Fisher Scientific). Mixture is ice cooled during sonication. Lysed cells are transfered into centrifugation tubes and centrifuged at 15,000 rpm for 20 minutes using JA17 rotor at 4 C. Discard supernatant and weigh pellets. Again, store pellets at -80 until next procedure.

RECONSTITUTION:

Thoroughly resuspend the pellets in 6M Guanidine + 50 mM HEPES (Research Organics) + 200 mM Na_2SO_4 buffer (pH 7.0). 2 to 3 ml guanidine buffer per gram pellet (mixture may be sonicated again to help resuspend pellet).

Mixture is transferred to a 250 ml Schlenk flask containing a magnetic stirrer. Connect septa stoppered flask to a vacuum-argon manifold system. Apply vacuum for 2-3 minutes and flush with argon for several cyles to reduce oxygen content. Add B-2-Mercaptoethanol (10 ul per gram pellet) to flask and stir for 15-20 minutes.

While mixture is stirring, prepare 0.14 M ferrous ammonium sulfate solution using the same anaerobic Tris buffer taking care that the ferrous state is not oxidized to the ferric state. 0.1ml 0.14 M ferrous sulfate solution per gram pellet is transferred anaerobically to the schlenck flask. After addition of ammonium sulfate, the solution is diluted dropwise overnight with approximately 200 ml anaerobic Tris buffer.

Separate reaction mixture by centrifugation at 15,000 rpm 15 minutes at 4 C again using JA17 rotor. The Hr protein should now be solubilized in the supernatant which is indicated by a pink color.

PURIFICATION:

The pink colored supernatant is then transferred into an Amicon stirred cell equipped with a YM30 membrane and concentrated to 1/5 to 1/10 its original volume. If precipitate occurs, centrifuge the protein, wash membrane with dd water. Redilute the protein one in ten with precooled 50 mM HEPES + 150 nM Na_2SO_4 buffer (pH 7.5) and reconcentrate via Amicon. Repeat redilution/concentration procedure two more times. The pink protein obtained is nonfunctional protein.

In order to obtain functional Hr protein, the pink colored (oxyHr) protein needs to be oxidized with potassium ferricyanide (PFC). The amount of ferricyanide is dependent on the concentration of the oxyHr. Add a 50 fold excess of PFC to the oxyHr and stir at room temp for two hours. Dilute with precooled 50 mM HEPES + 150 mM Na_2SO_4 buffer and Amicon to a small volume. Redilute and reconcentrate until the eluting solution becomes completely colorless. The Hr is now yellow in color and is referred to as MetHr. Concentration of metHR is determined by spectra. Ab at 355nm. MetHr concentration should be A(355)xDf/6.4 (mM).

The yellow colored metHr is next reduced by Sodiun Dithionite (SD). This is accomplished by dialyzing metHr against SD solution in 50 mM HEPES + 150 mM Na_2SO_4 buffer. To reduce metHr: (1) boil membrane tubing in 50 mM Tris + 2 mM EDTA for 5 minutes and rinse with distilled water. (2) in a large fask with stirrer, degas 50 mM HEPES +150 mM Na_2SO_4 buffer 100 x greater in volume than the metHr and flush 3 x with argon to assure solution is anaerobic. (3) clump one side of dialysis tubing, put metHr into tubing and seal opposite side. (4) weigh out 10x concentration SD and add protein in tubing and SD under argon to the flask. (5) degas and flush flask with argon. Stir at room temp for at least 8 hours or until yellow color inside the tubing becomes completely colorless. (6) Transfer the reduced protein in tubing to fresh, degassed 50 mM HEPES + 150 mM Na2SO4 buffer, stir at 4C for several hours (7) Transfer once more to fresh buffer under aerobic conditions. Protein is oxidized to functional oxyHr.

Protein purity is checked by SDS gel and spectra. If contaminants still remain, superose-12 column purification is needed. Elution buffer is the same as Amicon step. Fractions representing pure protein are collected. Finally, purified protein is diluted with Kreb's Buffer and concentrated to a small volume.

Krebs Buffer

3400 ml H_2O
100 ml each of six stock solutions
8 g dextrose
400 ul EDTA stock solution EDTA 10^{-5} in final buffer

	stock solutions	conc in final buffer
NaCl	275.9 g L	118 mM
KCl	14.0 g L	4.7 mM
$NaHCO_3$	84.0 g L	25 mM
$MgSO_4$	5.78g L	1.2 mM
KH_2PO_4	5.99 g L	1.1 mM
$CaCl_2.2H_2O$	14.7 g L	2.5 mM

Optional indomethacin (inhibitor of prostaglandin synthesis)

106 mg Na_2CO_3 in 10 ml H_2O
Add 200 mg indomethacin
100 ml per liter of buffer

VASCULAR RING STUDIES

Crossections of arteries are utilized to determine any vasoconstrictive or dilatory effects of hemerythrin. Coronary dog arteries and human femoral arteries were suspended in tissue baths aerated with Krebs Henseleit solution and equilibrated. Optimal resting tension was determined for each ring by the maximal response to norepinephrine. Response of rings to acetocholine and Prostaglandin $f_2$alpha is determined.

Canine arteries

Initial protein concentration before addition to 10 ml tissue baths 11mg/ml

amts added incrementally to each bath

10 ul, 25 ul, 50 ul, 100 ul, 250 ul, 500 ul

no response

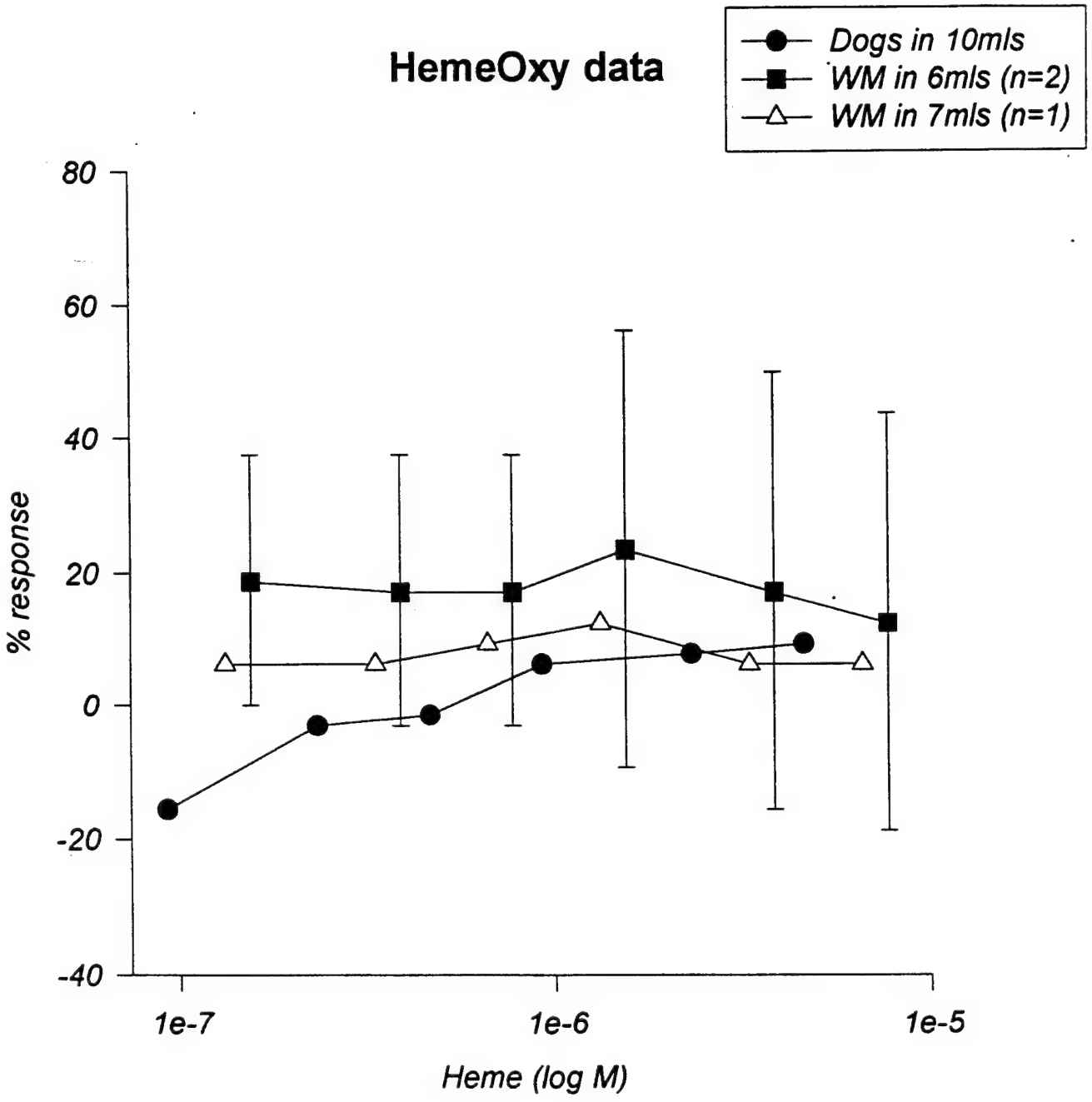
Human arteries

Initial protein concentration before addition to tissue 6 and 7 ml tissue baths 10 mg/ml

amts added incrementally to each bath

10 ul, 25 ul, 50 ul, 100ul, 250 ul, 500 ul

no response


HemeOxy data
Dogs in 10mls
WM in 6mls (n=2)
WM in 7mls (n=1)
80
60
40
20
0
-20
-40
% response
1e-7
1e-6
1e-5
Heme (log M)

Tissue	Fred			
PT #	The Dog			
R/G	Animal	7 ml		
Date	S-10-25-95	A-10-26-95		

Ring 1		Ring 2		Av70mMKCL
1 70mM KC	3.4	1 70mM KC	2.7	
2 70mM KC	3.6	2 70mM KC	3.1	3.5
Max Cn KC	3.5	Max Cn KCL		

Ring1	Ring2
70 Mm	70 Mm
Ten(gms)	Ten(gms)
1.6	1.6
%KCL	%KCL
45.71429	45.71429

Heme ml	Ten(gms)	Ten(gms)	Avg Ten	%RLXkcL	AvgTen
					AvgKCL
					45.71429
0.01	1.9	0.8	1.35	-15.625	9.3E-08
0.025	2.1	1	1.55	-3.125	2.3E-07
0.05	2.1	1.05	1.575	-1.5625	4.6E-07
0.1	2.3	1.1	1.7	6.25	9.3E-07
0.25	2.3	1.15	1.725	7.8125	2.3E-06
0.5	2.2	1.3	1.75	9.375	4.6E-06

Fun Endo ? NO

Tissue	SV					Tissue	SV
PT #	221-721					PT #	478-633
R/G	WM		6ml			R/G	WM
Date	S-01-10-96	A-01-12-96				Date	S-01-08-96
Ring 1		Ring 2		Av70mMKCL		Ring 1	
1 70mM KC	1.3	1 70mM KC	1.5	----------		1 70mM KC	1.25
2 70mM KC	1.45	2 70mM KC	2.2	3.225		2 70mM KC	1.4
Max Cn KC	1.375	Max Cn KC	1.85			Max Cn KC	1.325
Ring1	Ring2					Ring1	Ring2
70 Mm	70 Mm					70 Mm	70 Mm
Ten(gms)	Ten(gms)	AvgTen				Ten(gms)	Ten(gms)
1.6	1.6	1.6				1.6	1.6
%KCL	%KCL	AvgKCL				%KCL	%KCL
49.6124	49.6124	49.6124				120.7547	120.7547

Heme ml	Ten(gms)	Ten(gms)	Avg Ten	%RLXkcL		Heme ml	Ten(gms)
0.01	1.6	1.6	1.6	0	1.5E-07	0.01	2.2
0.025	1.5	1.6	1.55	-3.125	3.9E-07	0.025	2.2
0.05	1.5	1.6	1.55	-3.125	7.7E-07	0.05	2.2
0.1	1.3	1.6	1.45	-9.375	1.5E-06	0.1	2.5
0.25	1.2	1.5	1.35	-15.625	3.9E-06	0.25	2.4
0.5	1.2	1.4	1.3	-18.75	7.7E-06	0.5	2.3

Fun Endo ?	NO	NO	Fun Endo ?	NO

6ml

A-01-12-96

Ring 2	Av70mMKCL
1 70mM KCl	
2 70mM KCL	1.325
Max Cn KCL	

AvgTen
1.6

AvgKCL
120.7547

Ten(gms)	Avg Ten	%RLXkcL	
	2.2	37.5	1.5E-07
	2.2	37.5	3.9E-07
	2.2	37.5	7.7E-07
	2.5	56.25	1.5E-06
	2.4	50	3.9E-06
	2.3	43.75	7.7E-06

Tissue SV
PT # 478-633
R/G WM 7 ml
Date S-01-08-96 A-01-12-96

Ring 1		Ring 2
1 70mM KC	0.8	1 70mM KCl
2 70mM KC	0.9	2 70mM KCL
Max Cn KC	0.85	Max Cn KCL

Ring1	Ring2
70 Mm	70 Mm
Ten(gms)	Ten(gms)
1.6	1.6
%KCL	%KCL
188.2353	188.2353

Heme ml	Ten(gms)	Ten(gms)	Avg Ten
0.01	1.7		1.7
0.025	1.7		1.7
0.05	1.75		1.75
0.1	1.8		1.8
0.25	1.7		1.7
0.5	1.7		1.7

Ring 1	Ring 2	AvgTen
		1.6
		AvgKCL
		188.2353

Fun Endo ? NO

=========
Av70mMKCL

0.85

========
%RLXkcL

6.25	1.3E-07
6.25	3.3E-07
9.375	6.6E-07
12.5	1.3E-06
6.25	3.3E-06
6.25	6.6E-06